

**PATENT APPLICATION  
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**MULTIVALENT ANTIBODY CONSTRUCTS**

**Inventors:**      **Melvyn Little  
Sergej Kipriyanov**

**PENNIE & EDMONDS LLP  
1155 Avenue of the Americas  
New York, New York 10036-2711**

**(650) 493-4935**

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This is a national phase filing of the Application No. PCT/DE99/01350, which was filed with the Patent Corporation Treaty on May 5, 1999, and is entitled to priority of the German Patent Application 198 19 846.9, filed May 5, 1998.

**10 I. FIELD OF THE INVENTION**

The present invention relates to multivalent  $F_v$  antibody constructs, expression plasmids which code for them, and a method for producing the  $F_v$  antibody constructs as well as the use thereof.

**15 II. BACKGROUND OF THE INVENTION**

Natural antibodies are dimers and are therefore referred to as bivalent. They have four variable domains, namely two  $V_H$  domains and two  $V_L$  domains. The variable domains serve as binding sites for an antigen, a binding site being formed from a  $V_H$  domain and a  $V_L$  domain. Natural antibodies recognize one antigen each, so that they are also referred to as  
20 monospecific. Furthermore, they also have constant domains which add to the stability of the natural antibodies. On the other hand, they are also co-responsible for undesired immune responses which result when natural antibodies of various animal species are administered mutually.

In order to avoid such immune responses, antibodies are constructed which lack the  
25 constant domains. In particular, these are antibodies which only comprise the variable domains. Such antibodies are designated  $F_v$  antibody constructs. They are often available in the form of single-chain monomers paired with one another.

However, it showed that  $F_v$  antibody constructs only have little stability. Therefore, their usability for therapeutic purposes is strongly limited.

30

Thus, it is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

According to the invention this is achieved by the subject matters defined in the  
5 claims.

### III. SUMMARY OF THE INVENTION

The present invention relates to a multivalent F<sub>v</sub> antibody construct having at least four variable domains which are linked with each other via the peptide linkers 1, 2 and 3.

- 10 The invention also concerns expression plasmids which code for such an F<sub>v</sub> antibody construct and a method of producing the F<sub>v</sub> antibody constructs as well as their use.

### IV. BRIEF DESCRIPTION OF THE DRAWINGS

- FIGURE 1 shows the genetic organization of an F<sub>v</sub> antibody construct (A) according  
15 to the invention and schemes for forming a bivalent (B) or tetravalent F<sub>v</sub> antibody construct (C). Ag: antigen; His<sub>6</sub>: six C-terminal histidine residues; stop: stop codon (TAA); V<sub>H</sub> and V<sub>L</sub>: variable region of the heavy and light chains.

- FIGURE 2 shows the scheme for the construction of the plasmids pDISC3x19-LL and pDISC3x19-SL. c-myc: sequence coding for an epitope which is recognized by the  
20 antibody 9E1, His<sub>6</sub>: sequence which codes for six C-terminal histidine residues; PelB: signal peptide sequence of the bacterial pectate lyase (PelB leader); rbs: ribosome binding site; Stop: stop codon (TAA); V<sub>H</sub> and V<sub>L</sub>: variable region of the heavy and light chains.

- FIGURE 3 shows a diagram of the expression plasmid pDISC3x19-LL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for  
25 an epitope which is recognized by the 9E10 antibody; ColE1: origin of the DNA replication; fl; Lac P/Of: wt lac-operon promoter/operator; linker 1: sequence which codes for a GlyGly dipeptide linking the V<sub>H</sub> and V<sub>L</sub> domains; linker 2: sequence coding for a (Gly<sub>4</sub>Ser)<sub>4</sub> polypeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of  
30 the bacterial pectate lyase; rbs: ribosome binding site; V<sub>H</sub> and V<sub>L</sub>: variable region of the heavy and light chains.

FIGURE 4 shows a diagram of the expression plasmid pDISC3x19-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for  $\beta$ -lactamase which is responsible for the ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope recognized by the 9E10 antibody; ColE1: origin of DNA replication; 5 f1-IG: intergenic region of the bacteriophage f1; Lac P/Of: wt lacoperon promoter/operator; linker 1: sequence which codes for a GyGly dipeptide which links the  $V_H$  and  $V_L$  domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide which links the hybrid scFv fragments; Pe1-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site;  $V_H$  and  $V_L$ : variable region of the heavy and light chains.

10 FIGURE 5 shows the nucleotide sequence and the amino acid sequence derived therefrom of the bivalent  $F_v$  antibody construct encoded by the expression plasmid pDIS3x19-LL. c-myc epitope: sequence coding for an epitope which is recognized by the antibody 9E10; CDR: region determining the complementarity; framework: framework region; His6 tail: sequence which codes for six C-terminal histidine residues; Pe1B leader: 15 signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site;  $V_H$  and  $V_L$ : variable region of the heavy and light chains.

FIGURE 6 shows the nucleotide sequence and the derived amino acid sequence of the tetravalent  $F_v$  antibody construct encoded by the expression plasmid pDISC3x19-SL. c-myc epitope: sequence coding for an epitope which is recognized by the 9E10 antibody; 20 CDR: region determining complementarity; framework: framework region; His6 tail: sequence coding for the six C-terminal histidine residues; Pe1B leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site;  $V_H$  and  $V_L$ : variable region of the heavy and light chains.

FIGURE 7 shows the nucleotide sequence and the derived amino acid sequence of a 25 connection between a gene which codes for an  $\alpha$ -factor leader sequence and a gene coding for the tetravalent  $F_v$  antibody construct in the *Pichia* expression plasmid pPIC-DISC-SL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae*- $\alpha$  factor secretion signal;  $V_H$ : variable region of the heavy chain. Rhombs indicate the signal cleaving sites.

30 FIGURE 8 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene coding for an  $\alpha$ -factor leader sequence and a gene which codes

for the bivalent F<sub>v</sub> antibody construct in the *Pichia* expression plasmid pPIC-DISC-LL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae*- $\alpha$  factor secretion signal; V<sub>H</sub>: variable region of the heavy chain. Rhombs show the signal cleaving sites.

5           FIGURE 9 shows a diagram of the expression plasmid pDISC5-LL. 6xHis: sequence coding for six C-terminal histidine residues; bla: gene which codes for  $\beta$ -lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilizing DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon-  
10 promoter/operator; LacZ': gene which codes for the  $\alpha$ -peptide of  $\beta$ -galactosidase; linker 1: sequence which codes for a GlyGly dipeptide connecting the V<sub>H</sub> and V<sub>L</sub> domains; linker 2: sequence which codes for a (Gly<sub>4</sub>Ser)<sub>4</sub> polypeptide linking the hybrid scFv fragments; M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pe1-B leader; signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding  
15 site which originates from the *E. coli* LacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; V<sub>H</sub> and V<sub>L</sub>: variable region of the heavy and light chains.

          FIGURE 10 shows a diagram of the expression plasmid pDISC6-SL. 6xHis:  
20 sequence which codes for six C-terminal histidine residues; bla: gene which codes for  $\beta$ -lactamase responsible for ampicillin resistance; bp: base pairs: c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilized DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon promoter/operator; LacZ': gene which codes for the  $\alpha$ -peptide of  $\beta$ -galactosidase; linker 1:  
25 sequence which codes for a Glygly dipeptide which links the V<sub>H</sub> and V<sub>L</sub> domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide linking the hybrid scFv fragments: M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pe1-B leader: signal peptide sequence of the bacterial pectate lyase; rbs:  
30 ribosome binding site originating from the *E. coli* lacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the

bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; tIPP: transcription terminator; V<sub>H</sub> and V<sub>L</sub>: variable region of the heavy and light chains.

## V. DETAILED DESCRIPTION OF THE INVENTION

5 It is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

Therefore, the subject matter of the present invention relates to a multivalent F<sub>v</sub> antibody construct which has great stability. Such a construct is suitable for diagnostic and  
10 therapeutic purposes.

The present invention is based on the applicant's insights that the stability of an F<sub>v</sub> antibody construct can be increased if it is present in the form of a single-chain dimer where the four variable domains are linked with one another via three peptide linkers. The applicant also recognized that the F<sub>v</sub> antibody construct folds with itself when the middle  
15 peptide linker has a length of about 10 to 30 amino acids. The applicant also recognized that the F<sub>v</sub> antibody construct folds with other F<sub>v</sub> antibody constructs when the middle peptide linker has a length of about up to 10 amino acids so as to obtain a multimeric, *i.e.*, multivalent, F<sub>v</sub> antibody construct. The applicant also realized that the F<sub>v</sub> antibody construct can be multispecific.

20 According to the invention the applicant's insights are utilized to provide a multivalent F<sub>v</sub> antibody construct which comprises at least four variable domains which are linked with one another via peptide linkers 1, 2 and 3.

The expression "F<sub>v</sub> antibody construct" refers to an antibody which has variable domains but no constant domains.

25 The expression "multivalent F<sub>v</sub> antibody construct" refers to an F<sub>v</sub> antibody which has several, but at least four, variable domains. This is achieved when the single-chain F<sub>v</sub> antibody construct folds with itself so as to give four variable domains, or folds with other single-chain F<sub>v</sub> antibody constructs. In the latter case, an F<sub>v</sub> antibody construct is given which has 8, 12, 16, etc., variable domains. It is favorable for the F<sub>v</sub> antibody construct to have four or eight variable domains, *i.e.*, it is bivalent or tetravalent (FIGURE 1).

30 Furthermore, the variable domains may be equal or differ from one another, so that he

antibody construct recognizes one or several antigens. The antibody construct preferably recognizes one or two antigens, *i.e.*, it is monospecific and bispecific, respectively. Examples of such antigens are proteins CD19 and CD3.

5 The expression "peptide linkers 1, 3" refers to a peptide linker adapted to link variable domains of an  $F_v$  antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linkers 1 and 3 may be equal or differ from each other. Furthermore, the peptide linker may have a length of about 0 to 10 amino acids. In the former case, the peptide linker is only a peptide bond from the COOH residue of one of the variable domains  
10 and the  $NH_2$  residue of another of the variable domains. The peptide linker preferably comprises the amino acid sequence GG.

The expression "peptide linker 2" refers to a peptide linker adapted to link variable domains of an  $F_v$  antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The  
15 peptide linker may also have a length of about 3 to 10 amino acids, in particular 5 amino acids, and most particularly the amino acid sequence GGPGS, which serves for achieving that the single-chain  $F_v$  antibody constructs. The peptide linker can also have a length of about 11 to 20 amino acids, in particular 15 to 20 amino acids, and most particularly the amino acid sequence  $(G_4S)_4$ , which serves for achieving that the single-chain  $F_v$  antibody  
20 construct folds with itself.

An  $F_v$  antibody constructs according to the invention can be produced by common methods. A method is favorable in which DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an  $F_v$  antibody construct such that the peptide linkers link the variable domains with one another and the resulting  
25 DNA molecule is expressed in an expression plasmid. Reference is made to Example 1 to 6. As to the expressions " $F_v$  antibody construct" and "peptide linker" reference is made to the above explanations and, by way of supplement, to Maniatis, T. *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.

DNAs which code for an  $F_v$  antibody construct according to the invention also  
30 represent a subject matter of the present invention. Furthermore, expression plasmids which contain such DNAs also represent a subject matter of the present invention. Preferred



expression plasmids are pDISC3x19-LL, pDISC3x19-SL, pPIC-DISC-LL, pPIC-DISC-SL, pDISC5-LL and pDISC6-SL. The first four were deposited with the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellen) [German-type collection for micro-organisms and cells] on April 30, 1998 under DSM 12150, DSM 12149, DSM 12152 and DSM 12151,

5 respectively.

Another subject matter of the present invention relates to a kit, comprising:

- (a) an F<sub>v</sub> antibody construct according to the invention, and/or
- (b) an expression plasmid according to the invention, and
- (c) conventional auxiliary agents, such as buffers, solvents and controls.

10 One or several representatives of the individual components may be present.

The present invention provides a multivalent F<sub>v</sub> antibody construct where the variable domains are linked with one another via peptide linkers. Such an antibody construct distinguishes itself in that it contains no parts which can lead to undesired immune reactions. Furthermore, it has great stability. It also enables to bind several antigens  
15 simultaneously. Therefore, the F<sub>v</sub> antibody construct according to the invention is perfectly adapted to be used not only for diagnostic but also for therapeutic purposes. Such purposes can be seen as regards any disease, in particular a viral, bacterial or tumoral disease.

The below examples explain the invention in more detail. The following  
20 preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition  
25 to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

30

## VI. EXAMPLES

### A. Example 1: Construction of the Plasmids Pdisc3x19-ll and Pdisc3x9-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F<sub>v</sub> Antibody Constructs in Bacteria

The plasmids pHOG- $\alpha$ CD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma HD37 which is specific to human CD19 (Kipriyanov *et al.*, 1996, *J.-Immunol. Meth.* 196:51-62) and from the hybridoma OKT3 which is specific to human CD3 (Kipriyanov *et al.*, 1997, *Protein Eng.* 10:445-453), respectively, were used for the construction of expression plasmids for a single-chain F<sub>v</sub> antibody construct. A PCR fragment 1 of the V<sub>H</sub> domain of anti-CD19, followed by a segment which codes for a GlyGly linker, was produced using the primers DP1, 5'-TCACACAGAATTC-  
TTAGATCTATTAAGAGGAGAAATTAACC, and DP2, 5'-  
AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC (FIGURE. 2). The PCR fragment 1 was cleaved by EcoRI and EcoRV and ligated with the EcoRI/EcoRV-linearized plasmid pHOG-dmOKT3 so as to produce the vector pHOG19-3. The PCR  
fragment 2 of the V<sub>L</sub> domain of anti-CD19, followed by a segment which codes for a c-myc epitope and a hexahistidiny tail, was produced using the primers DP3, 5'-  
AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAAC-TCCA, and DP4, 5'-AGCACACTCTAGAGACACACAGATCTTTAGTGATGGTGAT-  
GGTGATGTGAGTTTAGG. The PCR fragment 2 was cleaved by HindIII and XbaI and  
ligated with the HindIII/XbaI-linearized plasmid pHOG-dmOKT3 so as to obtain the vector pHOG3-19 (FIGURE 2). The gene coding for the hybrid scFv-3-19 in the plasmid pHOG3-19 was amplified by means of PCR with the primers Bi3sk, 5'-  
CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG and either Li-1, 5'-  
TATATACTGCAGCTGCACCTGGCTACCACCACCACCGGAGCCG-  
CCACCACCGCTACCACCGCCGCCAGAACCACCACCACCAGCGGCCGCAGCATC  
AGCCCG, for the production of a long flexible (Gly<sub>4</sub>Ser)<sub>4</sub> inter-scFV linker (PCR fragment 3, FIGURE 2) or Li-2, 5'-TATATA-  
CTGCAGCTGCACCTGCGACCCTGGGCCACCAGCGGCCGCAGCATCAGCCG, for  
the production of a short rigid GGPGS linker (PCR fragment 4, FIGURE 2). The  
expression plasmids pDISC3x19-LL and pDISC3x19-SL were constructed by ligating the  
NcoI/PvuII restriction fragment from pHOG19-3, comprising the vector framework and the

NcoI/PvuII-cleaved PCR fragments 3 and 4, respectively (FIGURES 3 and 4). The complete nucleotide and protein sequences of the bivalent and tetravalent F<sub>v</sub> antibody constructs are indicated in FIGURES 5 and 6, respectively.

5            **B.        Example 2: Construction of the Plasmids Ppic-disc-ll and Ppic-disc-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F<sub>v</sub> Antibody Constructs in Yeast**

**(A) Construction of pPIC-DISC-SL**

                 The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins in the yeast *Pichia pastoris* was used as a starting  
10        material. It contains a gene which codes for the *Saccharomyces cerevisiae* α-factor secretion signal, followed by a polylinker. The secretion of this vector is based on the dominant selectable marker, Zeocin™ which is bifunctional in both *Pichia* and *E. coli*. The gene which codes for the tetravalent F<sub>v</sub> antibody construct (scDia-SL) was amplified by means of PCR by the template pDIC3x19-SL using the primers 5-PIC, 5'-  
15        CCGTGAATTCCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGC, and pSEXBn 5'-GGTCGACGTTAACCGACAAACAACAGATAAAACG. The resulting PCR product was cleaved by EcoRI and XbaI and ligated in EcoRI/XbaI-linearized pPICZαA. The expression plasmid pPIC-DISC-SL was obtained. The nucleotide and protein sequences of the tetravalent F<sub>v</sub> antibody construct are shown in FIGURE 7.

20

**(B) Construction of pPIC-DISC-LL**

                 The construction of pPIC-DISC-LL was carried out on the basis of pPICZαA (Invitrogen BV, Leek, Netherlands) and pDISC3x19-LL (FIGURE 3). The plasmid -DNA pPICZαA was cleaved by EcoRI. The overhanging 5'-ends were filled using a Klenow  
25        fragment of the *E. coli* DNA polymerase I. The resulting DNA was cleaved by XbaI, and the large fragment comprising the pPIC vector was isolated. Analogous thereto the DNA of pDISC3x19-LL was cleaved by NcoI and treated with a Klenow fragment. Following the cleavage using XbaI a small fragment, comprising a gene coding for the bivalent F<sub>v</sub> antibody, was isolated. Its ligation with a pPIC-derived vector-DNA resulted in the plasmid  
30        pPIC-DISC-LL. The nucleotide and protein sequences of the bivalent F<sub>v</sub> antibody construct are shown in FIGURE 8.

**C. Example 3: Expression of the Tetravalent And/or Bivalent F<sub>v</sub> Antibody Construct in Bacteria**

*E. coli* XL1-blue cells (Stratagene, La Jolla, CA) which had been  
5 transformed with the expression plasmids pDISC3x19-L1 and pDISC3x19-SL, respectively,  
were cultured overnight in 2xYT medium with 50 µg/ml ampicillin and 100 mM glucose  
(2xYT<sub>GA</sub>) at 37°C. 1:50 dilutions of the overnight cultures in 2xYT<sub>GA</sub> were cultured as flask  
cultures at 37°C while shaking with 200 rpm. When the cultures had reached an OD<sub>600</sub>  
value of 0.8, the bacteria were pelleted by 10-minute centrifugation with 1500 g at 20°C and  
10 resuspended in the same volume of a fresh 2xYT medium containing 50 µg ampicillin and  
0.4 M saccharose. IPTG was added up to a final concentration of 0.1 mM, and the growth  
was continued at room temperature (20-22°C) for 18 - 20 h. The cells were harvested by  
10-minute centrifugation with 5000 g at 4°C. The culture supernatant was held back and  
stored on ice. In order to isolate the soluble periplasmic proteins, the pelleted bacteria were  
15 resuspended in 5 % of the initial volume of ice-cold 50 mM Tris\_HCl, 20 % saccharose, 1  
mM EDTA, pH 8.0. Following 1 hour of incubation on ice with occasional stirring the  
spheroplasts were centrifuged with 30,000 g at 4°C for 30 minutes, the soluble periplasmic  
extract being obtained as supernatant and the spheroplasts with the insoluble periplasmic  
material being obtained as pellet. The culture supernatant and the soluble periplasmic  
20 extract were combined and clarified by further centrifugation (30,000 g, 4°C, 40 min.). The  
recombinant product was concentrated by ammonium sulfate precipitation (final  
concentration 70 % saturation). The protein precipitate was obtained by centrifugation  
(10,000 g, 4°C, 40 min.) and dissolved in 10 % of the initial volume of 50 mM Tris-HCl, 1  
M NaCl, pH 7.0. An immobilized metal affinity chromatography (IMAC) was carried out at  
4°C using a 5 ml column of chelating sepharose (Pharmacia) which was charged with Cu<sup>2+</sup>  
25 and had been equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (starting buffer). The  
sample was loaded by passing it over the column. It was then washed with twenty column  
volume of starting buffer, followed by starting buffer with 50 mM imidazole until the  
absorption at 280 nm of the effluent was at a minimum (about thirty column volumes). The  
absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0.  
30

The protein concentrations were determined with the Bradford dye binding test (Bradford, 1976, *Anal. Biochem.* 72:248-254) using the Bio-Rad (Munich, Germany) protein assay kit. The concentrations of the purified tetravalent and bivalent F<sub>v</sub> antibody constructs were determined from the A<sub>280</sub> values using the extinction coefficients  $\epsilon^{1\text{mg/ml}} = 1.96$  and 1.93, 5 respectively.

**D. Example 4: Expression of the Tetravalent And/or Bivalent Antibody Construct in the Yeast *Pichia Pastoris***

Competent *P. pastoris* GS155 cells (Invitrogen) were electroporated in the 10 presence of 10  $\mu\text{g}$  plasmid-DNA of pPIC-DISC-LL and pPIC-DISC-SL, respectively, which had been linearized with SacI. The transformants were selected for 3 days at 30°C on YPD plates containing 100  $\mu\text{g}$  Zeocin™. The clones which secreted the bivalent and/or tetravalent F<sub>v</sub> antibody constructs were selected by plate screening using an anti-c-myc-mAK 9E10 (IC chemikalien, Ismaning, Germany).

For the expression of the bivalent F<sub>v</sub> antibody constructs and tetravalent F<sub>v</sub> antibody 15 constructs, respectively, the clones were cultured in YPD medium in shaking flasks for 2 days at 30°C with stirring. The cells were centrifuged resuspended in the same volume of the medium containing methanol and incubated for another 3 days at 30°C with stirring. The supernatants were obtained after the centrifugation. The recombinant product was 20 isolated by ammonium sulfate precipitation, followed by IMAC as described above.

**E. Examples 5: Characterization of the Tetravalent F<sub>v</sub> Antibody Construct and Bivalent F<sub>v</sub> Antibody Construct, Respectively**

**(A) Size exclusion chromatography**

An analytical gel filtration of the F<sub>v</sub> antibody constructs was carried out in PBS 25 using a superdex 200-HR10/30 column (Pharmacia). The sample volume and the flow rate were 200  $\mu\text{l/min}$  and 0.5 ml/min, respectively. The column was calibrated with high-molecular and low-molecular gel filtration calibration kits (Pharmacia).

**(B) Flow cytometry**

30 The human CD3<sup>+</sup>/CD19<sup>-</sup> -acute T-cell leukemia line Jurkat and the CD19<sup>+</sup>/CD3<sup>-</sup> B-cell line JOK-1 were used for flow cytometrie. 5 x 10<sup>5</sup> cells in 50  $\mu\text{l}$  RPMI 1640 medium

(GIBCO BRL, Eggestein, Germany) which was supplemented with 10 % FCS and 0.1 % sodium azide (referred to as complete medium) were incubated with 100  $\mu$ l of the F<sub>v</sub> antibody preparations for 45 minute on ice. After washing using the complete medium the cells were incubated with 100  $\mu$ l 10  $\mu$ g/ml anti-c-myc-Mak 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second wash cycle, the cells were incubated with 100  $\mu$ l of the FITC-labeled goat-anti-mouse-IgG (GIBCO BRL) under the same conditions as before. The cells were then washed again and resuspended in 100  $\mu$ l 1  $\mu$ g/ml propidium iodide solution (Sigma, Deisenhofen, Germany) in complete medium with the exclusion of dead cells. The relative fluorescence of the stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

*(C) Cytotoxicity test*

The CD19-expressing Burkitt lymphoma cell line Raji and Namalwa were used as target cells. The cells were incubated in RPMI (GIBCO BRL) which was supplemented with 10 % heat-inactivated FCS (GIBCO BRL), 2 mM glutamine and 1 mM pyruvate, at 37°C in a dampened atmosphere with 7.5 % CO<sub>2</sub>. The cytotoxic T-cell tests were carried out in RPMI-1640 medium supplemented with 10 % FCS, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. The cytotoxic activity was evaluated using a standard [<sup>51</sup>Cr] release test; 2 x 10<sup>6</sup> target cells were labeled with 200  $\mu$ Ci Na[<sup>51</sup>Cr]O<sub>4</sub> (Amersham-Buchler, Braunschweig, Germany) and washed 4 times and then resuspended in medium in a concentration of 5 x 10<sup>6</sup>/ml. Increasing amounts of CTLs in 100  $\mu$ l were titrated to 10<sup>4</sup> target cells/well or cavity in 50  $\mu$ l. 50  $\mu$ l antibodies were added to each well. The entire test was prepared three times and incubated at 37°C for 4 h. 100  $\mu$ l of the supernatant were collected and tested for [<sup>51</sup>Cr] release in a gamma counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). The maximum release was determined by incubation of the target cells in 10 % SDS, and the spontaneous release was determined by incubation of the cells in medium alone. The specific lysis (%) was calculated as: (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100.

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**F. Examples 6: Construction of the Plasmids Pdisc5-ll and Pdisc5-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F<sub>v</sub> Antibody Constructs in Bacteria by High Cell Density Fermentation**

Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system and a gene which codes for the *skp*/OmpH periplasmic factor for a greater production of recombinant antibodies. The *skp* gene was amplified by PCR using the primers *skp*-1, 5'-CGA ATT CTT AAG ATA AGA AGG AGT TTA TTG TGA AAA AGT GGT TAT TAG CTG CAG G and *skp*-2, 5'-CGA ATT AAG CTT CAT TAT TTA ACC TGT TTC AGT ACG TCG G using the plasmid pGAH317 (Holck and Kleppe, 1988, *Gene* 67:117-124). The resulting PCR fragment was cleaved by *Afl*III and *Hind*III and inserted in the *Afl*III/*Hind*III-linearized plasmid pHKK (Horn et al., 1996, *Appl. Microbiol. Biotechnol.* 46, 524-532) so as to obtain the vector pSKK. The genes obtained in the plasmids pDISC3x19-LL and pDISC3x19-SL and coding for the scFv antibody constructs were amplified by means of the primers *fe*-1, 5'-CGA ATT TCT AGA TAA GAA GGA GAA ATT AAC CAT GAA ATA CC and *fe*-2, 5'-CGA ATT CTT AAG CTA TTA GTG ATG GTG ATG GTG ATG TGA G. The *Xba*I/*Afl*III-cleaved PCR fragments were inserted in pSKK before the *skp* insert so as to obtain the expression plasmids pDISC5-LL and pDISC6-SL, respectively, which contain tri-cistronic operons under the control of the *lac* promoter/operator system (FIGURES 9 and 10).

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.